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(54) Title: DEFENSE-RELATED SIGNALING GENES AND METHODS OF USE

(57) Abstract: The invention relates to the genetic manipulation of plants, particularly to the expression of nucleotide sequences from defense-related signaling genes. Isolated nucleotide sequences encoding a neoxanthin cleavage enzyme, an amino acid permease, and a novel protein are provided. Also provided are isolated nucleotide sequences comprising promoters that drive expression in a plant in an inducible or tissue-preferred manner. The nucleotide sequences find use in increasing the resistance of plants to pathogens and other stresses, modifying ABA metabolism in plants, modifying amino acid transport and content in plants, and regulating gene expression in plants. Additionally provided are isolated proteins, and transformed plants and seeds thereof.

DEFENSE-RELATED SIGNALING GENES AND METHODS OF USE

FIELD OF THE INVENTION

The invention relates to the genetic manipulation of plants, particularly to transforming plants with genes that enhance disease resistance.

BACKGROUND OF THE INVENTION

Throughout their lives, plants are routinely subjected to a variety of stresses, which act to impede or alter growth and development processes. Stresses to plants may be caused by both biotic and abiotic agents. For example, biotic causes of stress include infection with a pathogen, insect feeding, and parasitism by another plant such as mistletoe, and even grazing by ruminant animals. Abiotic stresses include osmotic stress, excessive light intensity or insufficient light intensity, cold temperatures, warm temperatures, synthetic chemicals such as those used in agriculture, and excessive wind.

Because a stress negatively impacts plant growth and development processes, stress to agricultural plants has a negative economic impact expressed in the form of reduced yields, increased expenditures for pesticides, or both. Developing crop plants that are better able to tolerate or even avoid stresses is desirable and will most certainly improve agricultural productivity. Given the world's both increasing human population and diminishing land area available for agriculture, improving agricultural productivity is a paramount challenge. A thorough understanding of the mechanisms used by plants to avoid or tolerate stresses may help in the development of new strategies of improving the stress tolerance of agricultural plants.

In spite of the great frequency of stresses, plants survive, and often flourish. Plants are able to do this because of the evolution of a variety of internal and external mechanisms for avoiding or tolerating stress. For example, higher plants possess leaves with waxy, water-impermeable surfaces and pores known as stomata, which serve to allow the escape of water vapor during the process of transpiration. The periphery of the stomatal pores is lined with a pair of cells

known as guard cells, which control the aperture of the pore. By modifying their size and shape through a turgor-pressure-mediated process, the guard cells can completely block the pore when conditions are unfavorable for transpiration during, for example, periods of low soil-water availability. Such a stress-avoidance system allows a plant to survive conditions of water stress by reducing transpiration to nearly zero and preventing dehydration.

Plants also possess defense systems which prevent or help limit the stresses resulting from attacks by pathogens and insects. One well-known defense system against plant pathogens is known as systemic acquired resistance. Another defense system is the systemic induction of proteinase inhibitors following insect damage, which is usually referred to as the systemic wound response. In both of these defense systems, the initial impact of the pathogen or insect is transmitted *via* a signal or signals to other parts of the plant, resulting in the increased expression of genes encoding proteins that are directly or indirectly inhibitory to the invading organism. The associated, systemic increase in defense gene products is known to increase the resistance of the plant to both current and future stresses from pathogens and insects.

While certain components of the systems that plants use to respond to abiotic and biotic stresses are known, most components have yet to be elucidated. Uncovering the genetic components of such systems will provide plant breeders with new targets for crop improvement strategies.

SUMMARY OF THE INVENTION

Methods and compositions for expressing defense-related, signaling genes are provided. The compositions comprise nucleotide sequences from defense-related, signaling genes isolated from sunflower. The nucleotide sequences of the invention relate to sunflower genes encoding a neoxanthin cleavage enzyme (NCE), an amino acid permease (AAP) and a novel glutamic acid-rich protein designated GRP (previously designated GPR). The compositions of the invention find use in agriculture, particularly in methods for increasing the resistance of plants to pathogens, methods for modifying abscisic acid metabolism in a plant, methods for modifying amino acid transport in a plant, and methods for modifying the amino acid content of a plant. The methods comprise stably transforming the

genome of a plant with nucleotide sequences of the invention operably linked to a promoter that drives expression in a plant cell.

Methods for regulating gene expression in plants are provided. The methods comprise stably transforming a plant with a DNA construct comprising a promoter from either a sunflower NCE gene or a sunflower GRP gene operably linked to a second nucleotide sequence. Methods involving a promoter from the NCE gene find use in modulating the expression of a gene in a plant in response to pathogens, oxidative stress, oxidants, and defense-related signaling molecules such as, for example, jasmonic acid and salicylic acid. Methods involving a promoter from the GRP gene find use in preferentially expressing a gene in a plant stems.

Expression cassettes comprising sequences of the invention are provided. Additionally provided are transformed plants, plant tissues, plant cells, and seeds thereof. Isolated proteins encoded by the sequences of the invention are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides an alignment of the amino acid sequence of the sunflower neoxanthin cleavage enzyme with neoxanthin cleavage enzyme-like proteins from other plants. The proteins include GenBank Accession Numbers O49675 from *Arabidopsis thaliana* (SEQ ID NO: 20), O49895 from *Malus domestica* (SEQ ID NO: 21), O24023 from *Lycopersicon esculentum* (SEQ ID NO: 22), and O24592 from *Zea mays* (SEQ ID NO: 23). A star below the aligned sequences indicates that the amino acid at that position is conserved across all five sequences. A dash within a sequence denotes a gap introduced into the sequence to achieve optimal alignment with the other sequences.

Figure 2 provides an alignment of the amino acid sequence of the sunflower AAP with AAP-like proteins from other plants. The proteins include GenBank Accession Numbers O04459, O22719, and O24405 from *Arabidopsis thaliana* (SEQ ID NOs: 24-26, respectively), and Q40414 from *Nicotiana sylvestris* (SEQ ID NO: 27).

Figure 3 depicts the results of a Northern blot analysis to determine the effects of *Sclerotinia* infection on transcript levels of defense-signaling genes in SMF3 sunflower tissues. The transcript levels of genes encoding sunflower NCE

(upper panel), AAP (middle panel), and GRP (lower panel) were assessed. Lanes 1, 3, 5, and 7 contain RNA samples from control (uninoculated) sunflower plants or cultures. Lanes 2, 4, 6, and 8 represent RNA samples from sunflower plants or cultures inoculated with *Sclerotinia*. Lanes 1 and 2 contain RNA samples isolated from stems harvested on day 3. Lanes 3 and 4 contain RNA samples isolated from leaf tissues harvested on day 3. Lanes 5 and 6 contain RNA samples isolated from leaves harvested on day 5. Lanes 7 and 8 contain RNA samples isolated from non-infected and infected sunflower callus 12 hours after inoculation, respectively. For comparison, lanes 9 and 10 contain RNA samples isolated from leaves harvested from six-week-old SMF3 (lane 9) and oxox transgenic sunflower plants (lane 10). The numbers to the right of the panel indicate the CuraGen quantitative expression assay (RNA profiling) results. NCE was repressed in oxox plants by 21.5-fold, whereas AAP and GRP were induced 3- and 23-fold, respectively.

Figure 4 depicts the results of a Northern blot analysis of NCE gene transcript levels under different chemical treatments. Six-week-old sunflower plants were sprayed with oxalic acid (OA) (5 mM), H_2O_2 (5 mM), salicylic acid (SA) (5 mM), and jasmonic acid (JA) (45 μM in 0.1% ethanol). The leaf samples were collected at 0, 6, 12, and 24 hours after the applications and immediately frozen in liquid nitrogen. Twenty μg of total RNA was loaded in each sample lane. (CK, control)

Figure 5 depicts the results of a Northern blot analysis of GRP gene transcript levels in different tissues: Total RNA was isolated from five different plant tissues: young seed (Se), corolla tubes (C), stem (S), leaf (L), and root (R) tissues. RNA samples (20 μg) from control SMF3 plants (right) and oxox transgenic plants (left) were loaded in adjacent lanes for comparison.

Figure 6 depicts the results of a Northern blot analysis of NCE and PR5 gene transcript levels in SMF3 and oxox-transgenic leaf tissues at three developmental stages. Total RNA (20 μg/lane) isolated from non-transformed SMF3 leaf tissues (a) and from oxox-transgenic leaf tissues (b) harvested at four, six and eight weeks after planting was utilized. The lowest panel represents an RNA gel loading control which depicts the fluorescence of the 28S rRNA bands in the presence of ultraviolet radiation following ethidium bromide staining.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the isolation of defense-related signaling genes from sunflower. *Sclerotinia* is an economically important fungal pathogen of crops such as sunflower, canola (*Brassica* sp.), and soybean. Thus, resistance to this pathogen is a highly desirably trait to incorporate into susceptible crop plants. Transgenic sunflower plants that constitutively express a gene encoding oxalate oxidase (oxox) display enhanced resistance to *Sclerotinia*. See copending U.S. Application Serial No. 60/053,123 filed July 18, 1997, herein incorporated by reference. Although the nature of this resistance phenomenon is unclear, it is known that the constitutive expression of a gene encoding oxox in sunflower causes the expression of other genes to be altered. The isolation, identification, and methods of use for three of these defense-related genes from sunflower is disclosed herein.

Methods and compositions for expressing defense-related, signaling genes are provided. The methods and compositions find use in agriculture, particularly in the breeding of crop plants with improved agronomic traits. The compositions comprise nucleotide sequences, and the proteins encoded thereof, from defense-related, signaling genes isolated from sunflower. The nucleotide sequences of the invention relate to sunflower genes encoding a neoxanthin cleavage enzyme (NCE) (SEQ ID NO: 1), an amino acid permease (AAP) (SEQ ID NO: 4), and a novel protein which has been designated GRP (SEQ ID NO: 6). Coding sequences are provided for each of the three sunflower genes set forth in SEQ ID NOs: 1, 4, and 6. The amino acid sequences set forth in SEQ ID NOs: 2, 5, and 7 disclose the sequences of the proteins encoded by the nucleotide sequences set forth in SEQ ID NOs: 1, 4, and 6, respectively. Additionally provided are nucleotide sequences comprising the promoters of an NCE gene (SEQ ID NO: 3) and a GRP gene (SEQ ID NO: 8).

Methods are provided for increasing the resistance of a plant to a pathogen. The methods comprise incorporating into the genome of a plant a DNA construct comprising a nucleotide sequence of the invention operably linked to a promoter that drives expression in a plant cell. The nucleotide sequences of the invention are selected from plant defense-signaling nucleotide sequences. Such nucleotide sequences include those encoding an NCE, AAP, or a GRP. Depending on the

desired outcome, the nucleotide sequence of the invention may be operably linked to a promoter for the transcription of either sense or antisense transcripts. Furthermore, it is recognized that fragments of the nucleotide sequences of the invention may used in methods for increasing disease resistance in a plant. Such a fragment may encode a domain or portion of a protein that when expressed in a plant increases the disease resistance of the plant. Alternatively, such a fragment may be operably linked to a promoter for the transcription of antisense transcripts. While the choice of promoter depends on the desired level, timing, and localization of expression, the preferred promoters for operably linking to the nucleotide sequences of the invention are constitutive, pathogen-inducible, and wound-inducible promoters.

While the methods of invention may be used to increase the resistance of a plant to any pathogen, the preferred pathogens are those that cause plant diseases. More preferably, the pathogens are plant-disease-causing fungi, bacteria, and viruses. Most preferably, the pathogens are *Sclerotinia* spp. that cause plant diseases.

NCE is involved in the biosynthesis of the plant signal molecule, abscisic acid (ABA) (Giraudat *et al.* (1994) *Plant Mol. Biol.* 26:1557-77; Schwartz *et al.* (1997) *Science* 276:1872-1874). NCE catalyzes the cleavage of neoxanthin into xanthoxin in the biosynthetic pathway leading to ABA. Originally identified as positive regulator of leaf abscission, ABA is a ubiquitous plant signal molecule that is involved in a variety of physiological processes including seed development, dormancy, and germination, osmotic-stress responses and defense responses such as the induction of the wound-inducible proteinase inhibitors of tomato and potato (Zeevaart *et al.* (1988) *Ann Rev. Plant Physiol. Plant Mol. Biol.* 39:439). During or immediately following a stress, ABA levels generally increase in plants. Stress-induced increases in ABA levels in plants are associated with stomatal closure, changes in gene expression, and plant adaptations to stress (Giraudat *et al.* (1994) *Plant Mol. Biol.* 26:1557-77; Schwartz *et al.* (1997) *Science* 276:1872-1874; Iturriaga *et al.* (1994) *Plant Mol. Biol.* 24:235-40). While the physiological role of ABA in signal transduction has not been determined, a recent report revealed that ABA functions through cyclic ADP-ribose in plants (Wu *et al.* (1998) *Science* 278:2126-30).

A first embodiment of invention involves increasing the resistance of a plant to a pathogen by decreasing the level of an NCE in the plant by transforming the plant with a nucleotide sequence corresponding to at least a portion of an NCE transcript operably linked to a promoter that drives expression in a plant. In sunflower, one or more NCE genes are expressed in leaves, and upon infection with *Sclerotinia*, the steady-state level of NCE transcripts in leaves is reduced relative to the level in uninfected leaves. Transgenic sunflower plants constitutively expressing a gene encoding oxalate oxidase display increased levels of resistance to *Sclerotinia* relative to the level of resistance of non-transgenic plants. Such transgenic also have a reduced level of NCE transcripts in their leaves relative to non-transgenic plants. Thus, reducing the level of NCE transcripts in a plant may also increase resistance in a plant. Preferably, the NCE nucleotide sequence is selected from the sequence set forth in SEQ ID NO: 1 and a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO. 2. While the choice of promoter depends on the desired timing, level, and localization of expression, the promoter is preferably selected from pathogen-inducible promoters and constitutive promoters. It is recognized that a native promoter from an NCE gene may also be utilized.

Long-distance transport of amino acids is mediated by several families of differentially expressed amino acid transporters. AAPs play important roles in amino acid transport (Hirner *et al.* (1999) *Plant J.* 14:535-44). AAPs are members of a family of integral membrane proteins that function as transporters of amino acids and peptides across membranes. There is now a great deal of physiological and biochemical evidence for specific amino acid carriers involved in the uptake and transport of different amino acids in plants (Hirner *et al.* (1999) *Plant J.* 14:535-44). The substrate specificity of these transporters varies. Some AAPs are specific for one or only a few amino acids. Others, however, are more promiscuous and are capable of transporting several different amino acids across membranes. The isolation of a cDNA encoding a sunflower AAP is disclosed herein. Also disclosed is that the gene encoding this AAP displays increased expression in transgenic plants constitutively expressing an oxox gene, relative to expression in similar, non-transgenic plants.

A second embodiment of invention involves increasing the resistance of a plant to a pathogen by increasing the level of an AAP in the plant by transforming the plant with a nucleotide sequence encoding an AAP operably linked to a promoter that drives expression in a plant. Transgenic sunflower plants constitutively expressing a gene encoding oxalate oxidase display increased levels of resistance to *Sclerotinia* relative to the level of resistance of non-transgenic plants. Such transgenic plants also have an increased level of AAP transcripts in their leaves relative to non-transgenic plants. It is recognized that increasing the transport of amino acids in a plant, such as transport mediated by AAPs, may increase the resistance of the plant to a pathogen by either increasing transport of a particular amino acid or class of amino acids.

Salicylic acid, a phenolic compound, is a key defense signal molecule in the well-known defense response phenomenon, systemic acquired resistance. The amino acid phenylalanine is a precursor in plants for the biosynthesis of a variety of defense-related molecules such as, for example, lignin, alkaloids, and phenolics including salicylic acid. Increasing the level in a plant of an AAP that transports phenylalanine may increase the level of phenylalanine available for salicylic acid biosynthesis and thus lead to an increased level of salicylic acid and increased disease resistance. Preferably, the increase in such an AAP occurs in a plant prior to, or at the time of, and in the vicinity of, the initial site of pathogen attack or infection.

Thus, the resistance of a plant to a pathogen may be increased by increasing the level of an AAP in the plant comprising transforming the plant with a nucleotide sequence encoding an AAP operably linked to a promoter that drives expression in a plant. The AAP nucleotide sequence is preferably selected from the sequence set forth in SEQ ID NO: 4 and a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 5. While the choice of promoter depends on the desired timing, level, and localization of expression, preferred promoters are pathogen-inducible promoters and constitutive promoters.

The isolation of a nucleotide sequence encoding a novel protein, designated GRP, is disclosed herein. Transgenic sunflower plants constitutively expressing a gene encoding oxalate oxidase display increased levels of resistance to *Sclerotinia* relative to the level of resistance of non-transgenic plants. Such transgenic plants

also have a increased level of GRP gene transcripts in their leaves relative to non-transgenic plants. Thus, GRP may play an important role in plant defense responses.

A third embodiment of the invention involves increasing the resistance of a plant to a pathogen by increasing the level of GRP in the plant comprising transforming the plant with a nucleotide sequence encoding GRP operably linked to a promoter that drives expression in a plant. Preferably, the GRP nucleotide sequence is selected from the sequence set forth in SEQ ID NO: 6 and a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 7. When expressed in a plant, such a GRP sequence increases the level of GRP in a plant. Thus, increasing the level of GRP in a plant may increase the resistance of the plant to a pathogen.

Methods for modifying ABA metabolism in a plant are provided. By "ABA metabolism" is intended metabolism that involves the metabolic pathways a plant uses to synthesize and degrade ABA, and by "ABA metabolite" is intended a metabolite of such a pathway. ABA is a plant growth regulator that is involved in a variety of physiological processes including growth inhibition and responses to biotic and abiotic stresses. By modifying ABA metabolism in a plant, the level of ABA can also be modified. Thus, the methods of the invention find use in modifying any physiological process in a plant that is affected by the endogenous level of ABA in the plant.

In addition, the methods for modifying ABA metabolism may be used to increase or decrease the level of one or more ABA metabolites. It is recognized that ABA metabolites include carotenoids, particularly xanthophylls and carotenes. Furthermore, certain carotenoids such as, for example, β-carotene and lycopene, are antioxidants that are marketed as nutraceuticals because they may help to protect the human body against certain forms of cancer. By modulating the levels of one or more enzymes in the ABA biosynthesis pathway, the levels of one or more ABA metabolites may also be modulated. Thus, the methods of the invention find use in producing intermediates associated with ABA metabolism.

By "modifying" or "modulating" is intended increasing or decreasing the level of a cellular component such as, for example, a protein, a transcript or a

metabolite, or the level of a cellular activity, such as for example, an enzyme activity, an activity of a transporter or an activity of a receptor.

The methods for modifying ABA metabolism in a plant comprise stably incorporating into the genome of a plant a nucleotide sequence encoding an enzyme involved in ABA metabolism operably linked to a promoter that drives expression in a plant. Preferably, such a nucleotide sequence encodes an NCE, a key enzyme in the ABA biosynthesis pathway. More preferably, such a nucleotide sequence encodes a sunflower NCE. Most preferably, such a nucleotide sequence is the nucleotide sequence set forth in SEQ ID NO: 1 or a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 2. While the choice of promoter depends on the desired timing, level and localization of expression, preferred promoters include constitutive, tissue-preferred, pathogen-inducible, wound-inducible, and chemically regulated promoters.

The methods for modifying ABA metabolism in a plant find use in increasing the tolerance of a plant to a stress. Increased levels of ABA in a plant are known to be associated with plant responses to abiotic and biotic stress. It is also known that such increased levels of ABA in a plant are involved in helping a plant to tolerate or recover from such stresses. Thus, increasing the level of ABA in a plant may increase the tolerance of a plant to a stress. Such a stress may be, for example, water stress, salt stress, heat stress, cold stress, stress caused by a pathogen, or stress caused by an insect.

A fourth embodiment of the invention involves increasing the level of ABA in a plant by increasing the level in a plant of a key enzyme in ABA biosynthesis, particularly NCE. To increase the level of ABA in a plant, the plant is transformed with a DNA construct comprising an NCE nucleotide sequence of the invention operably linked to a promoter that drives expression in a plant. As a key enzyme in the ABA biosynthesis pathway, increasing the level of NCE in a plant can increase the flux through the pathway in the direction of ABA, resulting in an increased level of ABA. The plant may be transformed with one of more additional DNA constructs comprising a nucleotide sequence encoding an enzyme in the ABA biosynthetic pathway operably linked to a promoter that drives expression in a plant. Such additional nucleotide sequences include, but are not limited to, those encoding the enzyme that catalyzes the conversion of xanthoxin to

ABA-aldehyde and the enzyme that catalyzes the conversion of ABA-aldehyde to ABA.

NCE possesses a putative chloroplast-targeting, transit peptide at its N-terminus. While NCE is not known to be localized to a particular organelle, it is expected to be found in chloroplasts and/or other plastids. In plants, carotenoids are predominantly found in plastids such as, for example, chloroplasts and chromoplasts. In addition, substrates of NCE, neoxanthin and violaxanthin, occur predominantly in the chloroplast. Furthermore, in green plant tissues, most free ABA is found in chloroplasts. Thus, it may be desirable to increase the level of NCE in chloroplasts, or other plastids.

There is evidence to show that abiotic response pathways such as, for example, wound response pathways, might also play a role in defense against specific fungal pathogens. The signaling molecules jasmonic acid and ethylene both regulate the expression of wound response genes and some basic PR genes in tobacco, and the defensin (PDF1.2) and thionin genes in *Arabidopsis* (Maleck and Dietrich (1999) *Trends Plant Sci.* 4:215-219). Herein disclosed is the expression of a gene encoding an NCE that is down-regulated by *Sclerotinia* infection, jasmonic acid, salicylic acid, and H_2O_2 treatments (Figures 3 and 4). Notably, the time course of down regulation in NCE gene expression closely coincided with the time course of up-regulation in PR5 gene expression (Figure 6). Thus, the *Sclerotinia* defense pathways may cross talk with the ABA biosynthesis pathway in a negative parallel manner in sunflower. Therefore, decreasing the level of ABA may increase the resistance to *Sclerotinia* in sunflower.

A fifth embodiment of the invention involves decreasing the level of ABA in a plant by eliminating, or reducing the level of, NCE in the plant. NCE is a key enzyme in ABA biosynthesis in plants. To lower the level of NCE in a plant, the plant is transformed with a DNA construct comprising a nucleotide sequence encoding at least a portion of an NCE transcript operably linked to a promoter in an orientation for the production of antisense NCE transcripts. Alternatively, a cosuppression approach may be employed to decrease production of NCE in the plant. By decreasing or eliminating NCE in a plant, the level of any one or more of ABA, ABA-aldehyde, xanthoxin, and ABA conjugates may be reduced in the plant. In addition, the levels of 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, substrates

of NCE, may be increased. Furthermore, blocking or inhibiting the flux of ABA metabolites in a plant toward the synthesis of ABA may lead to a buildup of one or more desirable carotenoids, including, but not limited to, xanthophylls, β-carotene and lycopene.

It is recognized that by reducing the level of NCE in a plant, the level of ABA, or any ABA metabolite between neoxanthin and ABA, may be reduced in the plant. It is further recognized that by lowering the level of an NCE in a plant, the level of neoxanthin, or any other ABA metabolite that occurs in the ABA biosynthesis pathway prior to NCE, may be increased in the plant.

Methods for modulating amino acid transport in a plant are provided. The methods find use in modulating the level and/or content of one or more amino acids in a plant, or in any cell, tissue, or organ thereof. By modulating in a plant the level of one or more amino acids necessary for the biosynthesis of a protein, the level of one or more proteins in the plant may be altered. The methods comprise transforming a plant with a nucleotide sequence encoding a protein that mediates the transport of amino acids across membranes, particularly biological membranes, operably linked to a promoter that drives expression in a plant. Preferably, such a nucleotide sequence encodes an AAP. More preferably, such a nucleotide sequence encodes a sunflower AAP. Most preferably, such a nucleotide sequence is the nucleotide sequence set forth in SEQ ID NO: 4 or a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 5. While the choice of promoter depends on the desired timing, level, and localization of expression, the promoter is preferably selected from constitutive, tissue-preferred, and seed-preferred promoters.

Methods are provided for modifying the amino acid content of a plant. The methods involve modifying a plant to produce an increased level of a protein which contains high percentages of the amino acids, glutamic acid and proline. The methods find use in agriculture for increasing the glutamic acid and/or proline content of a plant, particularly, a part of a plant that is harvested for human or animal consumption, more particularly a seed. The amino acid content of the GRP (SEQ ID NO: 7) disclosed herein comprises approximately 26.6% glutamic acid residues and 12.7% proline residues. The methods comprise stably incorporating in the genome of a plant a GRP nucleotide sequence of the invention operably

linked to a promoter capable of driving gene expression in a plant. Preferred GRP nucleotide sequences include the sunflower GRP nucleotide sequence set forth in SEQ ID NO: 6 and a nucleotide sequence encoding the amino acid sequence of a sunflower GRP set forth in SEQ ID NO: 7.

While a nucleotide sequence which encodes a native GRP may be used in the methods of the invention, it may be desirable to alter, preferably reduce, more preferably eliminate, the native GRP activity of the encoded GRP. Thus, a nucleotide sequence may be the modified to alter one or more amino acids in the encoded protein. The nucleotide sequences of the invention may be modified to increase the content of glutamic acid, proline, or both, in the encoded protein to levels higher than in found in the proteins encoded by the native sequences. Furthermore, fragments of the nucleotide sequences of the invention that encode protein domains with a particularly high level of glutamic acid or proline can be combined with other nucleotide sequences to increase the level of glutamic acid or proline in the encoded fusion protein. Alternatively, a nucleotide sequence fragment that encodes a protein domain having, for example, a particularly high level of glutamic acid can be used to synthesize a nucleotide sequence comprising multiple repeats of such a fragment. A protein encoded by repeats of such a nucleotide sequence encodes a glutamic acid-rich protein comprised of repeated, glutamic acid-rich domains. For example, a 27-amino-acid domain of GRP (amino acids 79-105 of SEQ ID NO: 7) possesses approximately 44% (12/27) glutamic acid residues.

Generally, it is desirable to direct the production of the GRP to a part of the plant that is harvested and/or is used for food or animal feed. While the choice of promoter depends on the desired timing, level, and localization of expression, the promoter is preferably selected from constitutive, tissue-preferred, and seed-preferred promoters. A GRP may be targeted to a specific cellular organelle, such as, for example, a protein body, by operably linking an appropriate targeting sequence to the nucleotide sequence encoding the GRP.

Methods for regulating gene expression in a plant in response to a stimulus are provided. The methods comprise transforming a plant with an inducible promoter operably linked to direct the expression of a nucleotide sequence of interest in response to a stimulus. The promoter is selected from a nucleotide

sequence of a promoter of a defense-signaling gene, particularly a promoter from an NCE gene. Preferably, such a promoter is from a sunflower NCE gene. More preferably, such a promoter is from a sunflower NCE gene that displays reduced gene expression in response to one or more stimuli including infection with a pathogen, damage from a pathogen, hydrogen peroxide, jasmonic acid, methyl jasmonate, salicylic acid, oxalic acid, and the expression of a gene encoding oxalic acid oxidase. Most preferably, such a promoter comprises the nucleotide sequence set forth in SEQ ID NO: 3.

While the methods of regulating gene expression may be used to regulate the expression of any nucleotide sequence of interest, the nucleotide sequence of interest is selected on the basis of the desired outcome. Generally, the nucleotide sequences of interest encode proteins or are antisense nucleotide sequences that correspond to transcripts from genes which encode proteins. Preferred nucleotide sequences of interest include defense activator nucleotide sequences. See copending U.S. Application Serial Numbers 09/256158 and 09/257541 both filed February 24, 1999; herein incorporated by reference.

In a sixth embodiment of the invention, methods are provided for regulating the expression of a defense activator nucleotide sequence involving operably linking a promoter of the invention to a defense activator nucleotide sequence. The methods of the invention find use in determining the role of defense activators in activating plant defense responses. The expression of such a defense activator nucleotide sequence in a plant may be used to induce the disease resistance pathway resulting in levels of immunity in the plant that impart resistance to the pathogen or induce cell death. However, it is unknown if such induced disease resistance requires the expression of a defense activator nucleotide sequence following the infection of a plant with a pathogen. The methods of the present invention involve the use of a promoter that down-regulates gene expression in a plant following infection of the plant with a pathogen. Thus, the expression of a defense activator that is operably linked to a promoter of the invention is down-regulated. Alternatively, if the defense activator nucleotide sequence is, for example, native to the genome of a plant, the methods of the invention may be used to suppress the expression of a native defense activator through antisense suppression. Such methods involve operably linking the

promoter of the invention to a defense activator nucleotide sequence in the antisense orientation for the production of antisense transcripts.

Methods are provided for stem-preferred regulation of gene expression in a plant. The methods involve transforming a plant with a DNA construct comprising a GRP promoter of the invention (SEQ ID NO: 8) operably linked to a nucleotide sequence. In both SMF3 and oxox transgenic sunflower plants, GRP transcript levels are significantly elevated in stems compared to the levels present in young seeds, corolla tubes, leaves, and roots, indicating that the GRP promoter preferentially drives expression in stems relative to the other tissues. Thus, the methods find use in directing stem-preferred expression of a desired nucleotide sequence in a plant.

While the methods of the invention can be used to drive the expression of any nucleotide sequence operably linked to a GRP promoter of the invention, preferred nucleotide sequences are those that improve at least one agronomically important trait of a crop plant including, but not limited to, nucleotide sequences that improve disease and/or insect resistance, increase stem strength, decrease stem height, and improve the nutritional value of stems for livestock feed. Of particular interest are nucleotide sequences which confer resistance to stem diseases including, but not limited to, *Sclerotinia* stem rot, *Phoma* black stem, and *Phomopsis* stem canker. For example, the GRP promoter can be operably linked to a nucleotide sequence encoding oxalate oxidase to improve the resistance of sunflower plants to *Sclerotinia*. Additionally, the GRP promoter can be used to drive the stem-preferred expression of nucleotide sequences encoding proteins with insecticidal activity against stem-damaging insects such as, for example, the European corn borer. Nucleotide sequences of interest that can be operably linked to the GRP promoter of the invention include, but are limited to, disease resistance genes, sequences encoding insecticidal proteins such as, for example, the well-known *Bacillus thuringiensis* (Bt) insecticidal proteins and sequences encoding enzymes or other proteins involved in gibberellin biosynthesis, degradation or action.

Compositions of the invention include isolated nucleotide sequences, isolated proteins, and expression cassettes that are related to defense-signaling genes. In particular, the present invention provides for isolated nucleic acid

molecules comprising nucleotide sequences encoding the amino acid sequences shown in SEQ ID NOs: 2, 5, and 7 or the DNA sequences deposited in a bacterial host as Patent Deposit Nos. PTA-78, PTA-79, and PTA-68 in the ATCC Patent Depository. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example those set forth in SEQ ID NOs: 1, 4, and 6, those deposited with the ATCC in a bacterial host as Patent Deposit Nos. PTA-78, PTA-79, and PTA-68, and fragments and variants thereof. Also provided are isolated nucleotide molecules comprising the promoter nucleotide sequences set forth in SEQ ID NOs: 3 and 8 which were deposited with the ATCC in a bacterial host as Patent Deposit Nos. Patent Deposit Nos. PTA-77 and PTA-1722, and fragments and variants thereof.

Plasmids containing the nucleotide sequences of the invention were deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, and assigned Patent Deposit Nos. PTA-68, PTA-77, PTA-78, PTA-79 and PTA-1722. PTA-77, PTA-78, and PTA-79 were deposited with the ATCC on May 13, 1999. PTA-68 was deposited with the ATCC on May 14, 1999. PTA-1722 was deposited with the ATCC on April 18, 2000. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. § 112.

The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid

molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein. For nucleotide sequences encoding an NCE, a fragment may encode a protein retaining NCE activity. For nucleotide sequences encoding an AAP, a fragment may encode a protein retaining AAP activity. For nucleotide sequences encoding a GRP, a fragment may encode a protein retaining the function of the native GRP protein. Fragments of a nucleotide sequence that are useful as hybridization probes may or may not encode fragment proteins retaining biological activity. Such fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length promoter sequence or full-length nucleotide sequence encoding the protein of the invention.

A fragment of an NCE nucleotide sequence that encodes a biologically active portion of a protein of the invention will encode at least 15, 25, 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, or 500 contiguous amino acids, or up to the total number of amino acids present in a full-length NCE protein of the invention (for example, 544 amino acids for SEQ ID NO: 2). Fragments of an NCE nucleotide sequence that are useful as hybridization probes for PCR primers generally need not encode a biologically active portion of an NCE protein.

Thus, a fragment of an NCE nucleotide sequence may encode a biologically active portion of an NCE. Such a fragment may also be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an NCE protein can be prepared by isolating a portion of an NCE

coding sequence of the invention, expressing the encoded portion of the NCE protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the portion of the NCE protein. Nucleic acid molecules that are fragments of an NCE coding sequence comprise at least 15, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, or 1,900 nucleotides, or up to the number of nucleotides present in a full-length NCE nucleotide sequence disclosed herein (for example, 1,950 nucleotides for SEQ ID NO: 1).

A fragment of an AAP nucleotide sequence that encodes a biologically active portion of a protein of the invention will encode at least 15, 25, 30, 50, 100, 150, 200, 250, 300, 350, 400, or 450 contiguous amino acids, or up to the total number of amino acids present in a full-length AAP protein of the invention (for example, 452 amino acids for SEQ ID NO: 5). Fragments of a AAP nucleotide sequence that are useful as hybridization probes for PCR primers generally need not encode a biologically active portion of a AAP protein.

Thus, a fragment of a AAP nucleotide sequence may encode a biologically active portion of an AAP protein. Such a fragment may also be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a AAP protein can be prepared by isolating a portion of one of the AAP coding sequences of the invention, expressing the encoded portion of the AAP protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the portion of the AAP protein. Nucleic acid molecules that are fragments of a AAP coding sequence comprise at least 15, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, or 1,600, nucleotides, or up to the number of nucleotides present in a full-length AAP nucleotide sequence disclosed herein (for example, 1,656 nucleotides for SEQ ID NO: 4).

A fragment of a GRP nucleotide sequence that encodes a biologically active portion of a protein of the invention will encode at least 15, 25, 30, 50, 100, or 150 contiguous amino acids, or up to the total number of amino acids present in a full-length GRP protein of the invention (for example, 173 amino acids for SEQ ID NO: 7). Fragments of a GRP nucleotide sequence that are useful as

hybridization probes for PCR primers generally need not encode a biologically active portion of a GRP protein.

Thus, a fragment of a GRP nucleotide sequence may encode a biologically active portion of an GRP protein. Such a fragment may also be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a GRP protein can be prepared by isolating a portion of one of the GRP coding sequences of the invention, expressing the encoded portion of the GRP protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the portion of the GRP protein. Nucleic acid molecules that are fragments of a GRP coding sequence comprise at least 15, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, or 850 nucleotides, or up to the number of nucleotides present in a full-length GRP nucleotide sequence disclosed herein (for example, 865 nucleotides for SEQ ID NO: 6).

Fragments and variants of the disclosed promoter sequences are also encompassed by the present invention. A fragment of an NCE promoter nucleotide sequence may retain the same promoter activity as the native promoter or may possess a promoter activity that is distinguishable from the native promoter. Fragments of a nucleotide sequence that are useful as hybridization probes may or may not retain promoter activity. Such fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length promoter sequence of the invention.

A biologically active portion of an NCE or GRP promoter can be prepared by isolating a portion of an NCE or GRP promoter sequence of the invention, making a DNA construct by operably linking the portion of the NCE or GRP promoter to a plant reporter gene, transforming a plant or plant cell with the DNA construct and assessing promoter activity by monitoring expression of the reporter gene. Nucleic acid molecules that are fragments of an NCE promoter sequence comprise at least 15, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, or 1,000 nucleotides, or up to the number of nucleotides present in NCE promoter nucleotide sequence disclosed herein (for example, 1,048 nucleotides for SEQ ID NO: 3). Nucleic acid molecules that are fragments of an GRP promoter sequence comprise at least 15, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000 1,100, or 1,200

nucleotides, or up to the number of nucleotides present in NCE promoter nucleotide sequence disclosed herein (for example, 1,267 nucleotides for SEQ ID NO: 8).

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the NCE, AAP, or GRP polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a NCE or AAP protein of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99%, or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, for example, NCE activity or AAP activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native NCE, AAP or GRP protein of the invention will have about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99%, or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as

few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the NCE, AAP, or GRP proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired NCE, AAP, or GRP activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity of NCE can be evaluated by ABA biosynthesis assays (see Tan *et al.* (1997) *Proc. Natl. Acad. Sci. USA 94*:12235-12240 and Schwartz *et al.* (1998) *Science 276*:1872; herein incorporated by reference) and the function of AAP can be evaluated by a yeast complementation assay (see, Chen and Bush

(1997) *Plant Physiol. 115*:1127-1134 and Rentsch *et al.* (1996) *Plant Cell 8*:1437-1446; herein incorporated by reference).

Variant proteins also encompass proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling (see U.S. Pat. No. 5,605,793 and 5,837,458). With such a procedure, one or more different NCE, AAP, or GRP coding sequences can be manipulated to create a new NCE, AAP, or GRP possessing the desired properties. Having identified the disclosed NCE, AAP, or GRP genes and the NCE, AAP, or GRP proteins encoded thereby, creation of new NCE, AAP, or GRP genes may be achieved with *in vitro* recombination of conserved motifs between NCE, AAP, or GRP genes. In this manner, sequence motifs encoding a domain of interest may be shuffled between an NCE, AAP, or GRP gene of the invention and other known NCE, AAP, or GRP genes. Strategies for such DNA shuffling to achieve domain swapping are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Moore *et al.* (1997) *J. Mol. Biol. 272*:336-347; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Crameri *et al.* (1998) *Nature 391*:288-291.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire NCE, AAP, or GRP sequences set forth herein, or to fragments thereof, are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant organism of interest. Methods for designing PCR primers

and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (*i.e.*, genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the NCE, AAP, or GRP sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire NCE, AAP, or GRP nucleotide disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding NCE, AAP, or GRP sequences and mRNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among NCE, AAP, or GRP sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding NCE, AAP, or GRP sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired plant organism or as a diagnostic assay to determine the presence of coding sequences in a plant an

organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30EC for short probes (e.g., 10 to 50 nucleotides) and at least about 60EC for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37EC, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55EC. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37EC, and a wash in 0.5X to 1X SSC at 55 to 60EC. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37EC, and a wash in 0.1X SSC at 60 to 65EC. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution.

For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth *et al.* (1984) *Anal. Biochem. 138*:267-284: T_m = 81.5EC + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1EC for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10EC. Generally, stringent conditions are selected to be about 5EC lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4EC lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10EC lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20EC lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45EC (aqueous solution) or 32EC (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

In general, isolated sequences that have promoter activity and which hybridize under stringent conditions to the NCE promoter sequences disclosed

herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 65% to 70% homologous, about 75% or 80% homologous, and even at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 40% to 50%, about 60%, 65%, or 70%, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity.

In general, isolated sequences that encode an NCE protein and which hybridize under stringent conditions to the NCE coding sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 65% to 70% homologous, about 75% or 80% homologous, and even at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 40% to 50%, about 60%, 65%, or 70%, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity.

In general, isolated sequences that encode for an AAP protein and which hybridize under stringent conditions to the AAP sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 70% to 75% homologous, about 80% or 85% homologous, and even at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 40% to 50%, about 60%, 65%, or 70%, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity.

In general, isolated sequences that have promoter activity and which hybridize under stringent conditions to the GRP promoter sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 60% to 65% homologous, about 70%, 75%, or 80% homologous, and even at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 40% to 50%,

about 60%, 65%, or 70%, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity.

In general, isolated sequences that encode for a GRP protein and which hybridize under stringent conditions to the GRP sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 60% to 65% homologous, about 70%, 75%, or 80% homologous, and even at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 40% to 50%, about 60%, 65%, or 70%, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS*

4:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* *2*:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. 85*:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 872*264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene 73*:237-244 (1988); Higgins *et al.* (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Res. 16*:10881-90; Huang *et al.* (1992) *CABIOS 8*:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol. 24*:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol. 215*:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs

(e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.hlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent

Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the

number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman *et al.* (1970) *J. Mol. Biol. 48*:443.

An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

The use of the term "DNA constructs" herein is not intended to limit the present invention to nucleotide constructs comprising DNA. Those of ordinary skill in the art will recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. Thus, the DNA constructs of the present invention encompass all nucleotide constructs which can be employed in the methods of the present invention for transforming plants including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The DNA constructs of the invention also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures and the like.

Furthermore, it is recognized that the methods of the invention may employ a DNA construct that is capable of directing, in a transformed plant, the expression of at least one protein, or at least one RNA, such as, for example, an rRNA, a tRNA and an antisense RNA that is complementary to at least a portion of an mRNA. Typically such a DNA construct is comprised of a coding sequence for a protein or an RNA operably linked to 5' and 3' transcriptional regulatory regions. Alternatively, it is also recognized that the methods of the invention may employ a DNA construct that is not capable of directing, in a transformed plant, the expression of a protein or RNA.

The DNA constructs of the invention also encompass nucleotide constructs, that may be employed in methods for altering or mutating a genomic nucleotide sequence in an organism, including, but not limited to, chimeric vectors, chimeric mutational vectors, chimeric repair vectors, mixed-duplex oligonucleotides, self-

complementary chimeric oligonucleotides and recombinogenic oligonucleobases. Such nucleotide constructs and methods of use, such as, for example, chimeraplasty, are known in the art. Chimeraplasty involves the use of such nucleotide constructs to introduce site-specific changes into the sequence of genomic DNA within an organism. See, U.S. Patent Nos. 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; all of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821 and Beetham *et al.* (1999) *Proc. Natl. Acad. Sci. USA 96*:8774-8778; herein incorporated by reference.

The NCE, AAP, or GRP nucleotide sequences of the invention are provided in expression cassettes for expression in the plant of interest. The cassette will include 5' and 3'-regulatory sequences operably linked to an NCE, AAP, or GRP coding sequence of the invention. Alternatively or additionally, the cassette will include an NCE or GPR promoter sequence of the invention and a 3'-regulatory sequence operably linked to a coding sequence. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the coding sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, an NCE, AAP, or GRP coding sequence of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence.

By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence, or any combination of a promoter with a coding sequence that is not identical to the structure of a native, unmodified gene.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used such as, for example, the NCE and GRP promoters of the invention. Such constructs would change expression levels of NCE, AAP, or GRP in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-158; Ballas *et al.* (1989) *Nuc. Acids Res. 17*:7891-7903; and Joshi *et al.* (1987) *Nuc. Acid Res. 15*:9627-9639.

Where appropriate, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the

host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *PNAS USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology* 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie et al. (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, tissue-preferred, or other promoters for expression in plants.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature 313*:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171);

ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18*:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730); ALS promoter (U.S. Application Serial No. 08/409,297), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

Generally, it will be beneficial to express the gene from an inducible promoter, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol. 89*:245-254; Uknes *et al.* (1992) *Plant Cell 4*:645-656; and Van Loon (1985) *Plant Mol. Virol. 4*:111-116. See also the copending applications entitled "Inducible Maize Promoters", U.S. Application Serial No. 60/076,100, filed February 26, 1998, and U.S. Application Serial No. 60/079,648, filed March 27, 1998, both of which are herein incorporated by reference.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol. 9*:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions 2*:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet. 2*:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA 93*:14972-14977. See also, Chen *et al.* (1996) *Plant J. 10*:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:2507-2511; Warner *et al.* (1993) *Plant J. 3*:191-201; Siebertz *et al.* (1989) *Plant Cell 1*:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path. 41*:189-200).

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the invention. Such wound-inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath. 28*:425-449; Duan *et al.* (1996) *Nature Biotechnology 14*:494-498); wun1 and wun2, U.S. Patent No. 5,428,148;

win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet. 215*:200-208); systemin (McGurl *et al.* (1992) *Science 225*:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol. 22*:783-792; Eckelkamp *et al.* (1993) *FEBS Letters 323*:73-76); MPI gene (Corderok *et al.* (1994) *Plant J. 6(2)*:141-150); and the like, herein incorporated by reference.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:10421-10425 and McNellis *et al.* (1998) *Plant J. 14(2)*:247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet. 227*:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to express the nucleotide sequences of the invention within a particular plant tissue. Leaf-preferred promoters include, Yamamoto *et al.* (1997) *Plant J. 12(2)*:255-265; Kawamata *et al.* (1997) *Plant Cell Physiol. 38(7)*:792-803; Hansen *et al.* (1997) *Mol. Gen. Genet. 254(3)*:337-343; Russell *et al.* (1997) *Transgenic Res. 6(2)*:157-168; Rinehart *et al.* (1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Lam (1994) *Results Probl. Cell Differ. 20*:181-196; Orozco *et al.* (1993) *Plant Mol. Biol. 23(6)*:1129-1138; Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90(20)*:9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505.

Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol. 20(2)*: 207-218 (soybean root-preferred glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell 3(10)*:1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol. 14(3)*:433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell 3(1)*:11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell 2(7)*:633-641, where two root-preferred promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) *79(1)*:69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J. 8(2)*:343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol. 29(4)*:759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol. 25(4)*:681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-preferred" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during

seed germination). See Thompson *et al.* (1989) *BioEssays 10*:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); and celA (cellulose synthase). Gama-zein is a preferred endosperm-specific promoter. Glob-1 is a preferred embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

Where low-level expression is desired, weak promoters will be used. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By low level is intended at levels of about 1/1000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Alternatively, it is recognized that weak promoters also encompass promoters that are expressed in only a few cells and not in others to give a total low level of expression. Where a promoter is expressed at unacceptably high levels, portions of the promoter sequence can be deleted or modified to decrease expression levels.

Such weak constitutive promoters include, for example, the core promoter of the Rsyn7 promoter (WO 99/43838 and U.S. Patent No. 6,072,050), the core 35S CaMV promoter, and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142. See also, copending application entitled "Constitutive Maize Promoters", U.S. Application Serial No. 60/076,075, filed February 26, 1998, and herein incorporated by reference.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech. 3*:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:6314-6318; Yao *et al.* (1992) *Cell 71*:63-72; Reznikoff (1992) *Mol. Microbiol.*

6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell 48*:555-566; Brown *et al.* (1987) *Cell 49*:603-612; Figge *et al.* (1988) *Cell 52*:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA 86*:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:2549-2553; Deuschle *et al.* (1990) *Science 248*:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol. 10*:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:5072-5076; Wyborski *et al.* (1991) *Nuc. Acids Res. 19*:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol. 10*:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother. 35*:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry 27*:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother. 36*:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature 334*:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

In some embodiments of the invention, the nucleic acids of interest are targeted to the chloroplast for expression. In this manner, where the nucleic acid of interest is not directly inserted into the chloroplast, the expression cassette will additionally contain a nucleic acid encoding a transit peptide to direct the nucleic acid of interest to the chloroplasts. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun. 196*:1414-1421; and Shah *et al.* (1986) *Science 233*:478-481.

Chloroplast-targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol. 30*:769-780; Schnell *et al.* (1991) *J. Biol. Chem. 266(5)*:3335-3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb. 22(6)*:789-810); tryptophan synthase (Zhao *et al.* (1995) *J. Biol. Chem. 270(11)*:6081-6087); plastocyanin

(Lawrence *et al.* (1997) *J. Biol. Chem. 272(33)*:20357-20363); chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem. 268(36)*:27447-27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem. 263*:14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun. 196*:1414-1421; and Shah *et al.* (1986) *Science 233*:478-481.

Methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA 90*:913-917; Svab and Maliga (1993) *EMBO J. 12*:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-preferred expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:7301-7305.

The nucleic acids of interest to be targeted to the chloroplast may be optimized for expression in the chloroplast to account for differences in codon usage between the plant nucleus and this organelle. In this manner, the nucleic acids of interest may be synthesized using chloroplast-preferred codons. See, for example, U.S. Patent No. 5,380,831, herein incorporated by reference.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile

Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London) 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that constitutive expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure constitutive expression of the desired phenotypic characteristic has been achieved.

The promoter nucleotide sequences and methods disclosed herein are useful for regulating the expression of any heterologous nucleotide sequence in a host plant in order to vary the phenotype of a plant. The NCE promoter sequences of the invention can be used to regulate the expression of any heterologous nucleotide sequence in a inducible manner. That is the NCE promoter nucleotide sequence directs increased transcription of the heterologous nucleotide sequence when the inducing agent or stimulus impacts a plant or a plant cell. Such stimuli include a pathogen, wounding, H_2O_2, jasmonic acid, methyl jasmonate, salicylic acid, methylsalicylic acid, oxalic acid and expression of a gene encoding oxalic acid oxidase. The GRP promoter can be used drive the stem-preferred expression of any heterologous nucleotide sequence. This the GRP promoter directs increased transcription of the heterologous nucleotide sequence preferentially in plant stems.

Various changes in phenotype are of interest including modifying the fatty acid composition in a plant, modulating the amino acid content of a plant, modulating a plant's pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterologous products or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes or cofactors in the plant. These changes result in a change in phenotype of the transformed plant.

Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and heterosis increase, the choice of genes for transformation will change accordingly. General categories of genes of interest include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and commercial products. Preferred genes of interest include those involved in insect and disease resistance.

Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example, *Bacillus thuringiensis* toxic protein genes (U.S. Patent Nos. 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; and Geiser *et al.* (1986) *Gene 48*:109); lectins (Van Damme *et al.* (1994) *Plant Mol. Biol. 24*:825); and the like.

Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (U.S. Application Serial No. 08/484,815, filed June 7, 1995); avirulence (avr) and disease resistance (R) genes (Jones *et al.* (1994) *Science 266*:789; Martin *et al.* (1993) *Science 262*:1432; and Mindrinos *et al.* (1994) *Cell 78*:1089); and the like.

By "antisense DNA nucleotide sequence" is intended a sequence that is in inverse orientation to the 5' to 3' normal orientation of that nucleotide sequence. When delivered into a plant cell, the antisense DNA sequence prevents normal expression of the DNA nucleotide sequence for the native gene. The antisense nucleotide sequence encodes an RNA transcript that is complementary to and capable of hybridizing to the endogenous messenger RNA (mRNA) produced by transcription of the DNA nucleotide sequence for the native gene. Expression of the antisense transcript RNA interferes with expression of the corresponding mRNA and hence disrupts production of the native protein.

It is recognized that with these nucleotide sequences, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for the NCE, AAP, or GRP sequences can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants.

Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation, also referred to as cosuppression methods, are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The present invention may be used for transformation of any plant species, including, but not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense, Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus*

spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), more preferably corn and soybean plants, yet more preferably corn plants.

Plants of particular interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, peanut, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

The invention is drawn to compositions and methods for inducing resistance in a plant to plant pests. Accordingly, the compositions and methods are also useful in protecting plants against fungal pathogens, viruses, nematodes, insects and the like.

By "disease resistance" is intended that the plants avoid the disease symptoms that are the outcome of plant-pathogen interactions. That is, pathogens are prevented from causing plant diseases and the associated disease symptoms, or alternatively, the disease symptoms caused by the pathogen is minimized or lessened. The methods of the invention can be utilized to protect plants from disease, particularly those diseases that are caused by plant pathogens.

Pathogens of the invention include, but are not limited to, viruses or viroids, bacteria, insects, nematodes, fungi, parasitic plants, and the like. Viruses include any plant virus, for example, tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, maize dwarf mosaic virus, etc. Specific fungal and viral

pathogens for the major crops include: Soybeans: *Phytophthora megasperma* fsp. *glycinea*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *sojae (Phomopsis sojae)*, *Diaporthe phaseolorum* var. *caulivora*, *Sclerotium rolfsii*, *Cercospora kikuchii*, *Cercospora sojina*, *Peronospora manshurica*, *Colletotrichum dematium (Colletotichum truncatum)*, *Corynespora cassiicola*, *Septoria glycines*, *Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. *glycinea*, *Xanthomonas campestris* p.v. *phaseoli*, *Microsphaera diffusa*, *Fusarium semitectum*, *Phialophora gregata*, Soybean mosaic virus, *Glomerella glycines*, Tobacco Ring spot virus, Tobacco Streak virus, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, Tomato spotted wilt virus, *Heterodera glycines Fusarium solani*; Canola: *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Mycosphaerella brassiccola*, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum*, *Alternaria alternata*; Alfalfa: *Clavibater michiganese subsp. insidiosum*, *Pythium ultimum*, *Pythium irregulare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var. *medicaginis*, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrochila medicaginis*, *Fusarium*, *Xanthomonas campestris* p.v. *alfalfae*, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*; Wheat: *Pseudomonas syringae* p.v. *atrofaciens*, *Urocystis agropyri*, *Xanthomonas campestris* p.v. *translucens*, *Pseudomonas syringae* p.v. *syringae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Collotetrichum graminicola*, *Erysiphe graminis* f.sp. *tritici*, *Puccinia graminis* f.sp. *tritici*, *Puccinia recondita* f.sp. *tritici*, *Puccinia striiformis*, *Pyrenophora tritici-repentis*, *Septoria nodorum*, *Septoria tritici*, *Septoria avenae*, *Pseudocercosporella herpotrichoides*, *Rhizoctonia solani*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis* var. *tritici*, *Pythium aphanidermatum*, *Pythium arrhenomanes*, *Pythium ultimum*, *Bipolaris sorokiniana*, Barley Yellow Dwarf Virus, Brome Mosaic Virus, Soil Borne Wheat Mosaic Virus, Wheat Streak Mosaic Virus, Wheat Spindle Streak Virus, American Wheat Striate Virus, *Claviceps purpurea*, *Tilletia tritici*, *Tilletia laevis*, *Ustilago*

tritici, *Tilletia indica*, *Rhizoctonia solani*, *Pythium arrhenomannes*, *Pythium gramicola*, *Pythium aphanidermatum*, High Plains Virus, European wheat striate virus; Sunflower: *Plasmophora halstedii*, *Sclerotinia sclerotiorum*, Aster Yellows, *Septoria helianthi*, *Phomopsis helianthi*, *Alternaria helianthi*, *Alternaria zinniae*, *Botrytis cinerea*, *Phoma macdonaldii*, *Macrophomina phaseolina*, *Erysiphe cichoracearum*, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus stolonifer*, *Puccinia helianthi*, *Verticillium dahliae*, *Erwinia carotovorum* pv. *carotovora*, *Cephalosporium acremonium*, *Phytophthora cryptogea*, *Albugo tragopogonis*; Corn: *Fusarium moniliforme* var. *subglutinans*, *Erwinia stewartii*, *Fusarium moniliforme*, *Gibberella zeae (Fusarium graminearum)*, *Stenocarpella maydi (Diplodia maydis)*, *Pythium irregulare*, *Pythium debaryanum*, *Pythium graminicola*, *Pythium splendens*, *Pythium ultimum*, *Pythium aphanidermatum*, *Aspergillus flavus*, *Bipolaris maydis* O, T *(Cochliobolus heterostrophus)*, *Helminthosporium carbonum* I, II & III *(Cochliobolus carbonum)*, *Exserohilum turcicum* I, II & III, *Helminthosporium pedicellatum*, *Physoderma maydis*, *Phyllosticta maydis*, *Kabatiella-maydis*, *Cercospora sorghi*, *Ustilago maydis*, *Puccinia sorghi*, *Puccinia polysora*, *Macrophomina phaseolina*, *Penicillium oxalicum*, *Nigrospora oryzae*, *Cladosporium herbarum*, *Curvularia lunata*, *Curvularia inaequalis*, *Curvularia pallescens*, *Clavibacter michiganense* subsp. *nebraskense*, *Trichoderma viride*, Maize Dwarf Mosaic Virus A & B, Wheat Streak Mosaic Virus, Maize Chlorotic Dwarf Virus, *Claviceps sorghi*, *Pseudonomas avenae*, *Erwinia chrysanthemi* pv. *zea*, *Erwinia carotovora*, *Corn stunt spiroplasma*, *Diplodia macrospora*, *Sclerophthora macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora maydis*, *Peronosclerospora sacchari*, *Sphacelotheca reiliana*, *Physopella zeae*, *Cephalosporium maydis*, *Cephalosporium acremonium*, Maize Chlorotic Mottle Virus, High Plains Virus, Maize Mosaic Virus, Maize Rayado Fino Virus, Maize Streak Virus, Maize Stripe Virus, Maize Rough Dwarf Virus; Sorghum: *Exserohilum turcicum*, *Colletotrichum graminicola (Glomerella graminicola)*, *Cercospora sorghi*, *Gloeocercospora sorghi*, *Ascochyta sorghina*, *Pseudomonas syringae* p.v. *syringae*, *Xanthomonas campestris* p.v. *holcicola*, *Pseudomonas andropogonis*, *Puccinia purpurea*, *Macrophomina phaseolina*, *Perconia circinata*, *Fusarium moniliforme*, *Alternaria alternata*, *Bipolaris sorghicola*,

Helminthosporium sorghicola, *Curvularia lunata*, *Phoma insidiosa*, *Pseudomonas avenae (Pseudomonas alboprecipitans)*, *Ramulispora sorghi*, *Ramulispora sorghicola*, *Phyllachara sacchari*, *Sporisorium reilianum (Sphacelotheca reiliana)*, *Sphacelotheca cruenta*, *Sporisorium sorghi*, Sugarcane mosaic H, Maize Dwarf Mosaic Virus A & B, *Claviceps sorghi*, *Rhizoctonia ~~solani~~*, *Acremonium strictum*, *Sclerophthona macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Sclerospora graminicola*, *Fusarium graminearum*, *Fusarium oxysporum*, *Pythium arrhenomanes*, *Pythium graminicola*, etc.

Nematodes include parasitic nematodes such as root knot, cyst, lesion, and reniform nematodes, etc.

Parasitic plants include, but are not limited to, true and dwarf mistletoes, witchweeds (*Striga spp.*), *Orobanche cumana* and other parasitic *Orobanche spp.*

Insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera. Insect pests of the invention for the major crops include: Maize: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, surgarcane borer; *Diabrotica virgifera*, western corn rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus spp.*, wireworms; *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn blot leafminer; *Anaphothrips obscrurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus urticae*, twospotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranea*,

granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes, Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*; corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella fusca*, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *zygogramma exclamationis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera murtfeldtiana*, sunflower seed midge; Cotton: *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm; *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*, bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Franklinkiella fusca*, tobacco thrips; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia includens*,

soybean looper; *Anticarsia gemmatalis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum hilare*, green stink bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*, seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestani*, strawberry spider mite; *Tetranychus urticae*, twospotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Delia platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Brevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, Flea beetle; *Mamestra configurata*, Bertha armyworm; *Plutella xylostella*, Diamond-back moth; *Delia* ssp., Root maggots.

The present invention also provides isolated nucleic acids comprising polynucleotides of sufficient length and complementarity to a gene of the invention to use as probes or amplification primers in the detection, quantification, or isolation of gene transcripts. For example, isolated nucleic acids of the present invention can be used as probes in detecting deficiencies in the level of mRNA in screenings for desired transgenic plants, for detecting mutations in the gene (e.g., substitutions, deletions, or additions), for monitoring up-regulation of expression or changes in enzyme activity in screening assays of compounds, for detection of any number of allelic variants (polymorphisms) of the gene, or for use as molecular markers in plant breeding programs. The isolated nucleic acids of the present invention can also be used for recombinant expression of polypeptides, or for use as immunogens in the preparation and/or screening of antibodies. The isolated nucleic acids of the present invention can also be employed for use in sense or antisense suppression of one or more genes of the invention in a host cell, tissue, or plant. Attachment of chemical agents, which bind, intercalate, cleave and/or crosslink to the isolated nucleic acids of the present invention can also be used to modulate transcription or translation. Further, using a primer specific to an

insertion sequence (e.g., transposon) and a primer which specifically hybridizes to an isolated nucleic acid of the present invention, one can use nucleic acid amplification to identity insertion sequence inactivated genes of the invention from a cDNA library prepared from insertion sequence mutagenized plants. Progeny seed from the plants comprising the desired inactivated gene can be grown to a plant to study the phenotypic changes characteristic of that inactivation. See, *Tools to Determine the Function of Genes*, 1995 Proceedings of the Fiftieth Annual Corn and Sorghum Industry Research Conference, American Seed Trade Association, Washington, D.C., 1995. Additionally, non-translated 5' or 3' regions of the polynucleotides of the present invention can be used to modulate turnover of heterologous mRNAs and/or protein synthesis. Further, the codon preference characteristic of the polynucleotides of the present invention can be employed in heterologous sequences, or altered in homologous or heterologous sequences, to modulate translational level and/or rates.

The present invention provides a method of genotyping a plant comprising a polynucleotide of the present invention. The plant may be a monocot, such as maize or sorghum, or alternatively, a dicot, such as sunflower or soybean. Genotyping provides a means of distinguishing homologues of a chromosome pair and can be used to differentiate segregants in a plant population. Molecular marker methods can be used for phylogenetic studies, characterizing genetic relationships among crop varieties, identifying crosses or somatic hybrids, localizing chromosomal segments affecting monogenic traits, map based cloning, and the study of quantitative inheritance. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Chapter 7, Clark, Ed., Springer-Verlag, Berlin (1997). For molecular marker methods, see generally, The DNA Revolution by Andrew H. Paterson 1996 (Chapter 2) in: Genome Mapping in Plants (ed. Andrew H. Paterson) by Academic Press/R. G. Landis Company, Austin, Texas, pp. 7-21.

The particular method of genotyping in the present invention may employ any number of molecular marker analytic techniques such as, but not limited to, restriction fragment length polymorphisms (RFLPs). Thus, the present invention further provides a means to follow segregation of a gene or nucleic acid of the present invention as well as chromosomal sequences genetically linked to these genes or nucleic acids using such techniques as RFLP analysis. Linked

chromosomal sequences are within 50 centiMorgans (cM), often within 40 or 30 cM, preferably within 20 or 10 cM, more preferably within 5, 3, 2, or 1 cM of a gene of the invention.

In the present invention, the nucleic acid probes employed for molecular marker mapping of plant nuclear genomes selectively hybridize, under selective hybridization conditions, to a gene encoding a polynucleotide of the present invention. Preferably, the probes are selected from polynucleotides of the present invention. Typically, these probes are cDNA probes or *Pst I* genomic clones. The length of the probes is discussed in greater detail, *supra*, but are typically at least 15 bases in length, more preferably at least 20, 25, 30, 35, 40, or 50 bases in length. Generally, however, the probes are less than about 1 kilobase in length. Preferably, the probes are single copy probes that hybridize to a unique locus in a haploid chromosome complement.

The present invention further provides a method of genotyping comprising the steps of contacting, under stringent hybridization conditions, a sample suspected of comprising a polynucleotide of the present invention with a nucleic acid probe. Generally, the sample is a plant sample; preferably, a sample suspected of comprising a maize polynucleotide of the present invention (e.g., gene, mRNA). The nucleic acid probe selectively hybridizes, under stringent conditions, to a subsequence of a polynucleotide of the present invention comprising a polymorphic marker. Selective hybridization of the nucleic acid probe to the polymorphic marker nucleic acid sequence yields a hybridization complex. Detection of the hybridization complex indicates the presence of that polymorphic marker in the sample. Preferably, the nucleic acid probe comprises a polynucleotide of the present invention.

The following examples are presented by way of illustration, not by way of limitation.

EXPERIMENTAL

EXAMPLE 1: Isolation of Sunflower Defense Signaling Genes

Materials and Methods

Plant materials

Sunflower plants were grown in the greenhouse and growth chamber. The sunflower line SMF3 and oxox-transgenic sunflower (line 193870) were used for mRNA profiling. Sunflower pathogen, *Sclerotinia sclerotiorum*, was maintained on PDA plates at 20°C in the dark.

For fungal infection and chemical treatments, SMF3 sunflower plants were planted in four-inch pots and grown in greenhouse for the first four weeks. After transfer to the growth chamber, the plants were maintained under 12-hour photoperiod at 22°C with an 80% relative humidity. Six-week old plants were inoculated with *Sclerotinia*-infected carrot plugs or sprayed with an aqueous solution of either 5 mM oxalic acid, 5 mM H_2O_2, 5 mM salicylic acid or 45 μM jasmonic acid (with 0.1% ethanol). For each plant, three petioles were inoculated and wrapped with a 1 inch x 2 inch piece of parafilm. Plant tissue samples were harvested at different time points and immediately frozen in liquid nitrogen and then stored at –80°C.

Preparation of total RNA for RNA profiling and Northern analysis

Plant materials were ground in liquid nitrogen, and total RNA was extracted by the Tri-Reagent method. RNA profiling was conducted using CuraGen Corporation's proprietary method sold under the trademark GENECALLING. See U.S. Patent No. 5,871,697, herein incorporated by reference. Profiles of RNA samples prepared from combined leaf and stem tissues of six-week-old oxox sunflower plants were compared to profiles of similar RNA samples from of six-week-old SMF3 plants.

Total RNA (20 μg) was separated in a 1% agarose gel containing formaldehyde. Ethidium bromide was included to verify equal loading of RNA. After transfer onto Hybond N+ membrane, the blots were hybridized with ^{32}P-labeled NCE, AAP, or GRP cDNA probes. As a control, a duplicate blot was hybridized with a probe complementary to 18S rRNA. Hybridization and washing conditions were performed according to Church *et al.* ((1984) *Proc. Natl. Acad. Sci. USA* 81:1991-1995).

Isolation of full-length or flanking sequences by PCR amplification of cDNA ends

The three defense-related cDNAs were isolated by using RNA profiling and PCR-based technologies. The sequence information generated by RNA

profiling was used for designing gene-specific primers for amplifying both 3'- and/or 5'-end regions of the target genes using the rapid amplification of cDNA ends (RACE) method. *Sclerotinia*-infected and oxox-induced sunflower cDNA libraries were directionally constructed into pBluescript phagemid using a ZAP-cDNA synthesis kit from Stratagene. The cDNA libraries were used as a source of templates for PCR amplification.

To facilitate cloning full-length cDNAs from the initially cloned regions, a pair of 26 bp vector primers P3 (SEQ ID NO: 9) and P4 (SEQ ID NO: 10) were designed to hybridize to pBluescript vector sequences flanking both the 3'- and 5'-end regions of the cloned cDNAs. Initially, the 5'-end vector primer was paired with a gene-specific primer (P1) to directionally amplify the 5' end of the cloned cDNA. Once the anticipated 5' end of a specific cDNA with an intact ATG start codon was cloned and sequenced, the full-length cDNA was amplified using a second gene-specific primer (P5) designed to hybridize to a sequence at or upstream of ATG region sequence and a 3'-end vector primer (P4). The 3' end of a target cDNA was amplified employing a gene-specific primer P2 and the 3' end vector primer, P4. PCR products were cloned and sequenced.

For the isolation of the NCE cDNA by PCR amplification, oligonucleotide primers P1, P2, and P5 (SEQ ID NOs: 11-13, respectively) were employed. For the isolation of the AAP cDNA, oligonucleotide primers P1 and P2 (SEQ ID NOs: 14-15 respectively) were utilized. Finally, oligonucleotide primers P1, P2, and P5 (SEQ ID NOs: 16-18, respectively) were utilized to isolate the GRP cDNA.

The PCR reactions were performed in a total volume of 25 μL in 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1 mM dNTPs; 0.25 μM of each primer; and 0.5 units of advantage cDNA polymerase mix (Clontech) or Pwo DNA polymerase (Boehringer). Genomic DNA and/or cDNA library mixtures were used as a source of templates for PCR amplification.

Isolation of promoters

The promoter region of an NCE gene was isolated from sunflower genomic DNA using the Universal GenomeWalker Kit (Clontech) according to the manufacturer's instruction using an oligonucleotide primer designed from the NCE cDNA (SEQ ID NO: 19). Restriction digested genomic DNAs were ligated with an adapter to construct pools of genomic DNA fragments for walking by PCR

(Siebert *et al.* (1995) *Nuc. Acids Res. 23*:1087-1088). The promoter region of a GRP gene was isolated using a similar approach.

Analysis of amplified PCR products

Amplified PCR fragments with the expected sizes were individually sliced out of a gel for use as template DNA for a second round of PCR amplification with the same conditions as the initial PCR reaction. Each second-round PCR product showing a single band with the expected size was cloned into TA vector (Clontech) according to the supplier's instructions. Identified positive clones were selected for DNA sequencing using an Applied Biosystems 373A (ABI) automated sequencer at the Nucleic Acid Analysis Facility of Pioneer Hi-Bred International, Incorporated. DNA sequence analysis was carried out with the Sequencer (3.0). Multiple-sequence alignments of the DNA sequence were carried out using Clustal W (Version 1.7, default parameters) within the bioinformatics software package from CuraGen Corporation sold under the trademark CURATOOLS.

Construction of *Sclerotinia*-infected and resistance-enhanced (oxox-induced) sunflower cDNA libraries

Six-week old SMF3 sunflower plants were infected with *Sclerotinia sclerotiorum* by petiole inoculation with *Sclerotinia*-infested carrot plugs. Six days after infection, leaf and stem tissues were collected from infected plants for total RNA isolation. Total RNA was also isolated from six-week-old sunflower oxox-transgenic plants (line 610255), which are known to express a wheat oxalate oxidase gene. Our previous studies indicated that elevated levels of H_2O_2, SA, and PR1 protein were detected in such oxox-transgenic plants at the six-week-old stage and these plants had a greater level of resistance to infection with *Sclerotinia* (see copending U.S. Application Serial Number 60/053,123 filed July 18, 1997). Messenger RNA was isolated using an mRNA purification kit (BRL) according to the manufacturer's instruction. The cDNA libraries were constructed with the ZAP-cDNA synthesis kit into pBluescript phagemid (Stratagene). A cDNA library mixture for PCR cloning was made from oxox transgenic stem and *Sclerotinia*-infected leaf libraries (1:2 mix).

Results

RNA profiling study of oxox-transgenic sunflower plants

Resistance to the fungal pathogen *Sclerotinia* is a trait of major importance for crops such as sunflower, canola (*Brassica* sp.), and soybean. Expression of oxox by constitutive promoters significantly enhances resistance to *Sclerotinia* in sunflower. In a growth chamber experiment, the oxox-transgenic sunflower plants, upon infection with *Sclerotinia* mycelia, were observed to have lesions that were one-sixth the size of lesions found on similarly treated, non-transformed plants. At the six-week-old stage, the oxox-transgenic sunflower plants displayed a lesion-mimic phenotype in mature leaves, indicating that oxox expression in sunflower induces a hypersensitive response. To identify the oxidase-induced genes and elucidate their roles in the enhanced resistance, an RNA profiling study was carried out. Using this approach, at least 30 bands were found to be induced, and at least 30 bands were found to be repressed in the oxox-transgenic stem and leaf tissues as compared to non-transformed SMF3 plants. The DNA fragments corresponding to three of the bands were isolated and sequenced, and the sequence information was used to clone the full-length clones.

Isolation of full-length, defense-related cDNAs from sunflower

A PCR-based cloning method was developed to efficiently isolate, from sunflower cDNA libraries, full-length cDNAs corresponding to plant defense genes. A cDNA library mixture containing both DNA from oxox-transgenic cDNA library and DNA from a *Sclerotinia*-infected cDNA library (1:2 mix) was used as template for PCR amplification. Using cDNA libraries as a source of template DNA in the PCR amplifications had two-fold benefits. First, the number of unexpected PCR products was reduced as compared with the use of genomic DNA as a source of templates. Second, the use of DNA templates from disease-induced cDNA libraries increased the chance of isolating defense-related genes.

NCE cDNA and its promoter

The NCE cDNA is 1950 bp in length (SEQ ID NO: 1) with an open reading frame of 544 amino acids (SEQ ID NO: 2). The calculated molecular mass is 59.3 kDa with pI at 5.57. The sunflower NCE shared 69% amino acid identity with an NCE-like protein from *Arabidopsis thaliana* (SEQ ID NO: 20) as determined by BLASTX (Version 2.0) using the default parameters (Figure 1). NCE is a key enzyme of ABA biosynthesis, which catalyzes the oxidative cleavage of 9-*cis*-

epoxy carotenoids to xanthoxin. Results from Northern blotting experiments indicated that the transcription of this gene was repressed in both oxox-transgenic and *Sclerotinia*-infected, sunflower leaf tissues (Figure 3). In addition, sunflower NCE gene expression was negatively regulated when sunflower plants were treated with chemicals involving disease signal transduction, such as H_2O_2 (5 mM), salicylic acid (SA) (5 mM), and jasmonic acid (JA) (45 μM in 0.1% ethanol), and with a pathogenic factor, oxalic acid (OA) (5 mM) (Figure 4).

Between the four-week-old and six-week-old stages, PR5 gene expression was significantly induced whereas NCE gene expression was dramatically repressed (Figure 6). These results indicate that there is a potential alteration in one or more pathways in the oxox-transgenic leaves between the four-week-old and six-week-old stages. While the exact role of the ABA pathway in pathogen defense responses has not yet been defined, the results with oxox sunflower reveal that down regulation of ABA biosynthesis pathway may promote the activation of the pathogen defense pathways in sunflower.

The promoter of the NCE gene was also isolated. Sequence analysis indicated that NCE promoter contains the pathogen-responsive elements, MRE and W-box (SEQ ID NO: 3). However, the W-box sequence (GGTCAA) in the sunflower NCE promoter was in a reverse orientation when compared to the orientation of W-boxes in other disease resistance genes. This reverse orientation may cause down-regulation effects.

AAP cDNA

The AAP cDNA is 1656 bp in length (SEQ ID NO: 4) with an open reading frame that encodes a protein of 452 amino acids (SEQ ID NO: 5). A calculated molecular mass of the deduced protein is 50.6 kDa with a pI at 8.5. The sunflower AAP shared 75% amino acid identity with an Arabidopsis AAP (SEQ ID NO: 24) as determined by BLASTX (Version 2.0) using the default parameters (Figure 2). Hydropathy analysis indicated that AAP is an integral membrane protein having eight, or possibly nine, putative, membrane-spanning domains. Northern blot analysis demonstrated that AAP was induced in oxox-transgenic sunflower plants. Two different sizes of AAP mRNA were detected in the control and *Sclerotinia* infected leaf tissues, indicating that there may be more than one AAP gene expressed in leaf tissues (Figure 3).

A cDNA encoding novel protein designated GRP

The GRP cDNA is 865 nucleotides in length (SEQ ID NO: 6) with an open reading frame that encodes a polypeptide of 173 amino acids (SEQ ID NO: 7). The polypeptide has a calculated molecular mass of 18.8 kDa and is very acidic with ~~pI of 3.92.~~ ~~The~~ polypeptide encoded by ~~the~~ GRP cDNA ~~has~~ has high percentage of both glutamic acid (46/173, 26.59%) and proline (22/173, 12.72%). Nucleotide sequence and amino acid sequence database searches did not reveal significant homology with any reported sequences. The GRP polypeptide shared 37% amino acid identity with a 144 amino acid region of a hypothetical 185.1 kDa protein from a *Synechocystis* sp. (SP-TrEMBL Accession No. P73032, SEQ ID NO: 28) as determined by BLASTX (Version 2.0) using the default parameters. Northern blot experiments (Figures 3 and 5) indicated that the gene corresponding to the GRP cDNA was highly expressed in stem tissue.

The promoter of the GRP gene was also isolated. Sequence analysis indicated that GRP promoter contains the pathogen-responsive element, W-box, and a bZIP protein-binding motif (SEQ ID NO: 8). Given the strong expression of GRP in stems relative to other tissues (Figure 5), the GRP promoter can be used to drive the expression of an operably linked nucleotide sequence in plant stems. Additionally, RNA profiling indicated that the GRP expression was increased 23-fold in oxox sunflower leaf tissues when compared to similar SMF3 plants. Thus, the GRP promoter can be used to drive the expression of an operably linked nucleotide sequence in plant tissues having increased oxalate oxidase expression.

EXAMPLE 2: Transformation and Regeneration of Sunflower Plants

Sunflower meristem tissues are transformed with an expression cassette containing the NCE, AAP, or GRP nucleotide sequence of the invention (see also European Patent Number EP 0 486233, herein incorporated by reference, and Malone-Schoneberg *et al.* (1994) *Plant Science 103*:199-207). Mature sunflower seed (*Helianthus annuus* L.) are dehulled using a single wheat-head thresher. Seeds are surface sterilized for 30 minutes in a 20% Clorox bleach solution with the addition of two drops of Tween 20 per 50 mL of solution. The seeds are rinsed twice with sterile distilled water.

Split embryonic axis explants are prepared by a modification of procedures described by Schrammeijer *et al.* (Schrammeijer *et al.*(1990) *Plant Cell Rep. 9*: 55-60). Seeds are imbibed in distilled water for 60 minutes following the surface sterilization procedure. The cotyledons of each seed are then broken off, producing a clean fracture at the plane of the embryonic axis. Following excision of the root tip, the explants are bisected longitudinally between the primordial leaves. The two halves are placed, cut surface up, on GBA medium consisting of Murashige and Skoog mineral elements (Murashige *et al.* (1962) *Physiol. Plant.*, *15*: 473-497), Shepard's vitamin additions (Shepard (1980) in *Emergent Techniques for the Genetic Improvement of Crops* (University of Minnesota Press, St. Paul, Minnesota), 40 mg/L adenine sulfate, 30 g/L sucrose, 0.5 mg/L 6-benzyl-aminopurine (BAP), 0.25 mg/L indole-3-acetic acid (IAA), 0.1 mg/L gibberellic acid (GA_3), pH 5.6, and 8 g/L Phytagar.

The explants are subjected to microprojectile bombardment prior to *Agrobacterium* treatment (Bidney *et al.* (1992) *Plant Mol. Biol. 18*: 301-313). Thirty to forty explants are placed in a circle at the center of a 60 X 20 mm plate for this treatment. Approximately 4.7 mg of 1.8 mm tungsten microprojectiles are resuspended in 25 mL of sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 1.5 mL aliquots are used per bombardment. Each plate is bombarded twice through a 150 mm nytex screen placed 2 cm above the samples in a PDS 1000® particle acceleration device.

Disarmed *Agrobacterium tumefaciens* strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains the NCE, AAP, or GRP nucleotide sequence is introduced into *Agrobacterium* strain EHA105 via freeze-thawing as described by Holsters *et al.* (1978) *Mol. Gen. Genet. 163*:181-187. This plasmid further comprises a kanamycin selectable marker gene (i.e, *nptII*). Bacteria for plant transformation experiments are grown overnight (28°C and 100 RPM continuous agitation) in liquid YEP medium (10 g/L yeast extract, 10 g/L Bactopeptone, and 5 g/L NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid maintenance. The suspension is used when it reaches an OD_{600} of about 0.4 to 0.8. The *Agrobacterium* cells are pelleted and resuspended at a final OD_{600}

of 0.5 in an inoculation medium comprised of 12.5 mM MES pH 5.7, 1 g/L NH_4Cl, and 0.3 g/L $MgSO_4$.

Freshly bombarded explants are placed in an *Agrobacterium* suspension, mixed, and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-cultivated, cut surface down, at 26°C and 18-hour days. After three days of co-cultivation, the explants are transferred to 374B (GBA medium lacking growth regulators and a reduced sucrose level of 1%) supplemented with 250 mg/L cefotaxime and 50 mg/L kanamycin sulfate. The explants are cultured for two to five weeks on selection and then transferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differentiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250 mg/L cefotaxime for a second 3-day phytohormone treatment. Leaf samples from green, kanamycin-resistant shoots are assayed for the presence of NPTII by ELISA. If, for example, the sunflower plants are transformed with an NCE, AAP, or GRP coding sequence, the presence of transgene expression may be determined by assaying for the level of the protein or a biological activity thereof.

NPTII-positive shoots are grafted to Pioneer® hybrid 6440 *in vitro*-grown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium (half-strength Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the transformed shoot inserted into the cut. The entire area is wrapped with parafilm to secure the shoot. Grafted plants can be transferred to soil following one week of *in vitro* culture. Grafts in soil are maintained under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T_0 plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by analysis of NCE, AAP, or GRP protein levels or biological activities in leaf extracts while transgenic seeds harvested from NPTII-positive T_0 plants are identified by analysis of NCE, AAP, or GRP protein levels or biological activities of small portions of dry seed cotyledon.

An alternative sunflower transformation protocol allows the recovery of

transgenic progeny without the use of chemical selection pressure. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 mL of solution, then rinsed three times with distilled water. Sterilized seeds are imbibed in the dark at 26°C for 20 hours on filter paper moistened with water. The cotyledons and root radical are removed, and the meristem explants are cultured on 374E (GBA medium consisting of MS salts, Shepard vitamins, 40 mg/L adenine sulfate, 3% sucrose, 0.5 mg/L 6-BAP, 0.25 mg/L IAA, 0.1 mg/L GA, and 0.8% Phytagar at pH 5.6) for 24 hours under the dark. The primary leaves are removed to expose the apical meristem, around 40 explants are placed with the apical dome facing upward in a 2 cm circle in the center of 374M (GBA medium with 1.2% Phytagar), and then cultured on the medium for 24 hours in the dark.

Approximately 18.8 mg of 1.8 μm tungsten particles are resuspended in 150 μL absolute ethanol. After sonication, 8 μL of it is dropped on the center of the surface of macrocarrier. Each plate is bombarded twice with 650 psi rupture discs in the first shelf at 26 mm of Hg helium gun vacuum.

The plasmid of interest is introduced into *Agrobacterium tumefaciens* strain EHA105 via freeze thawing as described previously. The pellet of overnight-grown bacteria at 28°C in a liquid YEP medium (10 g/L yeast extract, 10 g/L Bactopeptone, and 5 g/L NaCl, pH 7.0) in the presence of 50 μg/L kanamycin is resuspended in an inoculation medium (12.5 mM 2-mM 2-(N-morpholino) ethanesulfonic acid, MES, 1 g/L NH_4Cl, and 0.3 g/L $MgSO_4$ at pH 5.7) to reach a final concentration of 4.0 at OD 600. Particle-bombarded explants are transferred to GBA medium (374E), and a droplet of bacteria suspension is placed directly onto the top of the meristem. The explants are co-cultivated on the medium for 4 days, after which the explants are transferred to 374C medium (GBA with 1% sucrose and no BAP, IAA, GA3 and supplemented with 250 μg/mL cefotaxime). The plantlets are cultured on the medium for about two weeks under 16-hour day and 26 °C incubation conditions.

Explants (around 2 cm long) from two weeks of culture in 374C medium are screened for NCE, AAP, or GRP protein levels or biological activities using assays known in the art. After positive (i.e., for expression of the desired

transgene) explants are identified, those shoots that fail to exhibit the desired protein level or activity are discarded, and every positive explant is subdivided into nodal explants. One nodal explant contains at least one potential node. The nodal segments are cultured on GBA medium for three to four days to promote the formation of auxiliary buds from each node. Then they are transferred to 374C medium and allowed to develop for an additional four weeks. Developing buds are separated and cultured for an additional four weeks on 374C medium. Pooled leaf samples from each newly recovered shoot are screened again by the appropriate protein activity assay. At this time, the positive shoots recovered from a single node will generally have been enriched in the transgenic sector detected in the initial assay prior to nodal culture.

Recovered shoots positive for expression of the transgene of interest are grafted to Pioneer hybrid 6440 *in vitro*-grown sunflower seedling rootstock. The rootstocks are prepared in the following manner. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 mL of solution, and are rinsed three times with distilled water. The sterilized seeds are germinated on the filter moistened with water for three days, then they are transferred into 48 medium (half-strength MS salt, 0.5% sucrose, 0.3% gelrite pH 5.0) and grown at 26°C under the dark for three days, then incubated at 16-hour-day culture conditions. The upper portion of selected seedling is removed, a vertical slice is made in each hypocotyl, and a transformed shoot is inserted into a V-cut. The cut area is wrapped with parafilm. After one week of culture on the medium, grafted plants are transferred to soil. In the first two weeks, they are maintained under high humidity conditions to acclimatize to a greenhouse environment.

EXAMPLE 3: Soybean Embryo Transformation

Soybean embryos are bombarded with a plasmid containing the NCE, AAP, or GRP nucleotide sequence of the invention as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing

secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature* (London) *327*:70-73, U.S. Patent No. 4,945,050). A Du Pont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene 25*:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the NCE, AAP, or GRP nucleotide sequence of the invention can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µL of a 60 mg/mL 1 µm gold particle suspension is added (in order): 5 µL DNA (1 µg/µl), 20 µL spermidine (0.1 M), and 50 µL $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µL 70% ethanol and resuspended in 40 µL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed

approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 4: Transformation and Regeneration of Maize Plants

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the NCE, AAP, or GRP nucleotide sequence of the invention and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene 70*:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

A plasmid vector comprising NCE, AAP, or GRP nucleotide sequence of the invention is constructed. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten

pellets using a $CaCl_2$ precipitation procedure as follows:

100 µL prepared tungsten particles in water

10 µL (1 µg) DNA in Tris EDTA buffer (1 µg total DNA)

100 µL 2.5 M $CaCl_2$

10 µL 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 mL 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µL 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µL spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and

scored for the desired phenotypic trait associated with the NCE, AAP, or GRP nucleotide sequence of the invention.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 mL/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L thiamine HCl, 120.0 g/L sucrose, 1.0 mg/L 2,4-D, and 2.88 g/L L-proline (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 2.0 g/L Gelrite (added after bringing to volume with D-I H_20); and 8.5 mg/L silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 mL/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L thiamine HCl, 30.0 g/L sucrose, and 2.0 mg/L 2,4-D (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 3.0 g/L Gelrite (added after bringing to volume with D-I H_20); and 0.85 mg/L silver nitrate and 3.0 mg/L bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 mL/L MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/L thiamine HCL, 0.10 g/L pyridoxine HCL, and 0.40 g/L glycine brought to volume with polished D-I H_20) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/L myo-inositol, 0.5 mg/L zeatin, 60 g/L sucrose, and 1.0 mL/L of 0.1 mM abscisic acid (brought to volume with polished D-I H_20 after adjusting to pH 5.6); 3.0 g/L Gelrite (added after bringing to volume with D-I H_20); and 1.0 mg/L indoleacetic acid and 3.0 mg/L bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 mL/L MS vitamins stock solution (0.100 g/L nicotinic acid, 0.02 g/L thiamine HCL, 0.10 g/L pyridoxine HCL, and 0.40 g/L glycine brought to volume with polished D-I H_20), 0.1 g/L myo-inositol, and 40.0 g/L sucrose (brought to volume with polished D-I H_20 after adjusting pH to 5.6); and 6 g/L bacto-agar (added after bringing to volume with polished D-I H_20), sterilized and cooled to 60° C.

EXAMPLE 5: *Agrobacterium*-mediated Transformation of Maize

For *Agrobacterium*-mediated transformation of maize with an NCE, AAP, or GRP nucleotide sequence of the invention preferably the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the NCE, AAP, or GRP nucleotide sequence of the invention to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be

obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Applicant's or agent's file reference	5718-92-1	International application No.	PCT/US00/

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 16, lines 10, 14 and 15

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet ☐

Name of depository institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*
10801 University Blvd.
Manassas, VA 20110-2209 US

Date of deposit	Accession Number
13 May 1999 (13.05.99)	PTA-77

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INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

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Date of deposit	Accession Number
18 April 2000 (18.04.00)	PTA-1722

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THAT WHICH IS CLAIMED:

1. An isolated nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2;

(c) a nucleotide sequence comprising at least 21 contiguous nucleotides of a sequence of (a);

(d) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (a);

(e) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 4;

(f) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5;

(g) a nucleotide sequence comprising at least 30 contiguous nucleotides of a sequence of (e);

(h) a nucleotide sequence comprising at least 75% sequence identity to the sequence of (e);

(i) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 6;

(j) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 7;

(k) a nucleotide sequence comprising at least 21 contiguous nucleotides of a sequence of (i);

(l) a nucleotide sequence comprising at least 60% sequence identity to the sequence of (i);

(m) a nucleotide sequence that is the complement of the nucleotide sequence of any one of (a)-(l);

(n) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of any one of (a)-(m).

(o) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 3;

(p) a nucleotide sequence comprising at least 12 contiguous nucleotides of a sequence of (o);

(q) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (o);

(r) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 8;

(s) a nucleotide sequence comprising at least 15 contiguous nucleotides of a sequence of (r);

(t) a nucleotide sequence comprising at least 65% sequence identity to the sequence of (r);

(u) a nucleotide sequence that is the complement of the nucleotide sequence of any one of (o)-(t); and

(v) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of any one of (o)-(u).

2. An expression cassette comprising a nucleotide sequence of any one of (a)-(n) of claim 1, said nucleotide sequence operably linked to a promoter that drives expression in a plant cell.

3. The expression cassette of claim 2, wherein said second nucleotide sequence is a defense activator nucleotide sequence.

4. An expression cassette comprising the nucleotide sequence of any one of (o)-(u) of claim 1, said nucleotide sequence operably linked for expression to a second nucleotide sequence.

5. The expression cassette of claim 4, wherein said second nucleotide sequence encodes oxalate oxidase or a Bt toxin protein.

6. An isolated protein selected from the group consisting of:

(a) a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2;

(b) a polypeptide comprising at least 27 contiguous amino acids of the amino acid sequence of (a);

(c) a polypeptide comprising an amino acid sequence comprising at least 70% sequence identity to the sequence of (a);

(d) a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5;

(e) a polypeptide comprising at least 44 contiguous amino acids of the amino acid sequence of (d);

(f) a polypeptide comprising an amino acid sequence comprising at least 80 % sequence identity to the sequence of (d);

(g) a polypeptide comprising the amino acid set forth in SEQ ID NO: 7;

(h) a polypeptide comprising at least 6 contiguous amino acids of the amino acid sequence of (g);

(i) a polypeptide comprising an amino acid sequence comprising at least 50% sequence identity to the sequence of (g); and

(j) a polypeptide comprising a biologically active variant of the polypeptide of (a), (d), or (g).

7. A transformed plant comprising in its genome at least one stably incorporated DNA construct comprising a nucleotide sequence operably linked to a promoter that drives expression in a plant, wherein said nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2;

(c) a nucleotide sequence comprising at least 21

contiguous nucleotides of a sequence of (a);

(d) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (a);

(e) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 4;

(f) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5;

(g) a nucleotide sequence comprising at least 30 contiguous nucleotides of a sequence of (e);

(h) a nucleotide sequence comprising at least 75% sequence identity to the sequence of (e);

(i) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 6;

(j) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 7;

(k) a nucleotide sequence comprising at least 21 contiguous nucleotides of a sequence of (i);

(l) a nucleotide sequence comprising at least 60% sequence identity to the sequence of (i);

(m) a nucleotide sequence that is the complement of the nucleotide sequence of any one of (a)-(l); and

(n) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of any one of (a)-(m).

8. The plant of claim 7, wherein said promoter is selected from the group consisting of pathogen-inducible, wound-inducible, constitutive, tissue-preferred, and chemically regulatable promoters.

9. The plant of claim 7, wherein said nucleotide sequence is operably linked to said promoter for the production of antisense transcripts.

10. The plant of claim 7, wherein said plant is a monocot.

11. The plant of claim 10, wherein said monocot is selected from the group consisting of maize, wheat, rice, barley, sorghum, millet, and rye.

12. The plant of claim 7, wherein said plant is a dicot.

13. The plant of claim 12, wherein said dicot is selected from the group consisting of soybean, *Brassica* sp., sunflower, safflower, alfalfa, and cotton.

14. Transformed seed of the plant of claim 7.

15. A transformed plant cell comprising in its genome at least one stably incorporated DNA construct comprising a nucleotide sequence operably linked to a promoter that drives expression in a plant cell, wherein said nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2;

(c) a nucleotide sequence comprising at least 21 contiguous nucleotides of a sequence of (a);

(d) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (a);

(e) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 4;

(f) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5;

(g) a nucleotide sequence comprising at least 30 contiguous nucleotides of a sequence of (e);

(h) a nucleotide sequence comprising at least 75% sequence identity to the sequence of (e);

(i) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 6;

(j) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 7;

(k) a nucleotide sequence comprising at least 21 contiguous nucleotides of a sequence of (i);

(l) a nucleotide sequence comprising at least 60% sequence identity to the sequence of (i);

(m) a nucleotide sequence that is the complement of the nucleotide sequence of any one of (a)-(l); and

(n) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of any one of (a)-(m).

16. A method for increasing the resistance of a plant to a pathogen comprising stably incorporating in the genome of said plant a DNA construct comprising a nucleotide sequence operably linked to a promoter that drives expression in a plant, wherein said nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2;

(c) a nucleotide sequence comprising at least 21 contiguous nucleotides of a sequence of (a);

(d) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (a);

(e) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 4;

(f) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5;

(g) a nucleotide sequence comprising at least 30 contiguous nucleotides of a sequence of (e);

(h) a nucleotide sequence comprising at least 75% sequence identity to the sequence of (e);

(i) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 6;

(j) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 7;

(k) a nucleotide sequence comprising at least 21 contiguous nucleotides of a sequence of (i);

(l) a nucleotide sequence comprising at least 60% sequence identity to the sequence of (i);

(m) a nucleotide sequence that is the complement of the nucleotide sequence of any one of (a)-(l); and

(n) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of any one of (a)-(m);

wherein the resistance of said plant to said pathogen is increased.

17. The method of claim 16, wherein said promoter is selected from the group consisting of pathogen-inducible, wound-inducible, constitutive, tissue-preferred, and chemically regulatable promoters.

18. The method of claim 16, wherein said nucleotide sequence is operably linked to said promoter for the production of antisense transcripts.

19. The method of claim 16, wherein said pathogen is selected from the group consisting of a fungus, a virus, a bacterium, a nematode, and an insect.

20. A transformed plant comprising in its genome at least one stably incorporated DNA construct comprising a first nucleotide sequence operably to a second nucleotide sequence, wherein said first nucleotide sequence is a promoter that is capable of driving expression of said second nucleotide sequence in a plant and said first nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 3;

(b) a nucleotide sequence comprising at least 12 contiguous nucleotides of a sequence of (a);

(c) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (a);

(d) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 8;

(e) a nucleotide sequence comprising at least 15 contiguous nucleotides of a sequence of (d);

(f) a nucleotide sequence comprising at least 65% sequence identity to the sequence of (d);

(g) a nucleotide sequence that is the complement of the nucleotide sequence of any one of (a)-(f); and

(h) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of any one of (a)-(g).

21. The plant of claim 20, wherein said second nucleotide sequence is a defense activator nucleotide sequence or said second nucleotide sequence encodes oxalate oxidase or a Bt toxin protein.

22. Transformed seed of the plant of claim 20.

23. A transformed plant cell comprising in its genome at least one stably incorporated DNA construct comprising a first nucleotide sequence operably to a second nucleotide sequence, wherein said first nucleotide sequence is a promoter that is capable of driving expression of said second nucleotide sequence in a plant cell and said first nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 3;

(b) a nucleotide sequence comprising at least 12 contiguous nucleotides of a sequence of (a);

(c) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (a);

(d) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 8;

(e) a nucleotide sequence comprising at least 15 contiguous nucleotides of a sequence of (d);

(f) a nucleotide sequence comprising at least 65% sequence identity to the sequence of (d);

(g) a nucleotide sequence that is the complement of the nucleotide sequence of any one of (a)-(f); and

(h) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of any one of (a)-(g).

24. A method for regulating gene expression in a plant in response to a stimulus comprising stably incorporating into the genome of said plant a DNA construct comprising a first nucleotide sequence operably to a second nucleotide sequence, wherein said first nucleotide sequence is a promoter that is capable of driving expression of said second nucleotide sequence in a plant and said first nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 3;

(b) a nucleotide sequence comprising at least 12 contiguous nucleotides of a sequence of (a);

(c) a nucleotide sequence comprising at least 70% sequence identity to the sequence of (a); and

(d) a nucleotide sequence that hybridizes under stringent conditions to a sequence of (a);

wherein said stimulus is selected from the group consisting of infection with a pathogen, damage from a pathogen, hydrogen peroxide, jasmonic acid, methyl jasmonate, salicylic acid, oxalic acid and expression of a gene encoding oxalic acid oxidase.

25. The method of claim 24, wherein said second nucleotide sequence is a defense activator nucleotide sequence.

26. The method of claim 24, wherein said second nucleotide sequence is operably linked to said first nucleotide sequence for the production of antisense transcripts.

27. The method of claim 24, wherein the resistance of said plant to at least one pathogen is increased.

28. The method of claim 27, wherein said pathogen is selected from the group consisting of a fungus, a virus, a bacterium, a nematode, and an insect.

29. A method for stem-preferred regulation of gene expression in a plant comprising stably incorporating into the genome of said plant a DNA construct comprising a first nucleotide sequence operably to a second nucleotide sequence, wherein said first nucleotide sequence is a promoter that is capable of driving expression of said second nucleotide sequence in a plant and said first nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 8; and

(b) a nucleotide sequence comprising at least 15 contiguous nucleotides of a sequence of (a);

(c) a nucleotide sequence comprising at least 65% sequence identity to the sequence of (a); and

(d) a nucleotide sequence that hybridizes under stringent conditions to a sequence of (a);

wherein said second nucleotide sequence is preferentially expressed in a stem of said plant.

30. The method of claim 29, wherein said second nucleotide sequence encodes oxalate oxidase.

31. The method of claim 30, wherein the resistance of said plant to a pathogen is increased.

32. The method of claim 31, wherein said pathogen is selected from the group consisting of *Sclerotinia* spp., *Phoma* spp., and *Phomopsis* spp.

33. The method of claim 29, wherein said second nucleotide sequence is a disease resistance gene.

34. The method of claim 29, wherein said second nucleotide sequence encodes an insecticidal protein.

35. The method of claim 34, wherein said insecticidal protein is a Bt toxin protein.

36. The method of claim 34, wherein the resistance of said plant to at least one insect is increased.

37. The method of claim 36, wherein said insect is European corn borer.